Fungal Genetics and Biology 82 (2015) 43–50

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

The gene *ICS3* from the yeast *Saccharomyces cerevisiae* is involved in copper homeostasis dependent on extracellular pH

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ARTICLE INFO

Article history:
Received 9 February 2015
Revised 8 June 2015
Accepted 10 June 2015
Available online 27 June 2015

Keywords:
Copper homeostasis
Acidic conditions
Copper accumulation
pH dependent response

ABSTRACT

In the yeast *Saccharomyces cerevisiae*, many genes are involved in the uptake, transport, storage and detoxification of copper. Large scale studies have noted that deletion of the gene *ICS3* increases sensitivity to copper, Sortin 2 and acid exposure. Here, we report a study on the *Δics3* strain, in which *ICS3* is related to copper homeostasis, affecting the intracellular accumulation of this metal. This strain is sensitive to hydrogen peroxide and copper exposure, but not to other tested transition metals. At pH 6.0, the *Δics3* strain accumulates a larger amount of intracellular copper than the wild-type strain, explaining the sensitivity to oxidants in this condition. Unexpectedly, sensitivity to copper exposure only occurs in acidic conditions. This can be explained by the fact that the exposure of *Δics3* cells to high copper concentrations at pH 4.0 results in over-accumulation of copper and iron. Moreover, the expression of *ICS3* increases in acidic pH, and this is correlated with *CCC2* gene expression, since both genes are regulated by Rim101 from the pH regulon. *CCC2* is also upregulated in *Δics3* in acidic pH. Together, these data indicate that *ICS3* is involved in copper homeostasis and is dependent on extracellular pH.

1. Introduction

Copper and other transition metals are essential micronutrients, because of their catalytic and structural functions as prosthetic groups in metalloproteins. A variety of enzymes require copper as a cofactor for electron transfer reactions (Freitas et al., 2003); however, when in excess, metal ions produce toxic effects. Free metal ions can catalyse superoxide anion radical ($O_2^{\cdot-}$) formation, which leads to the formation of hydrogen peroxide (H_2O_2). Hydrogen peroxide reacts with free iron or copper to generate the highly reactive hydroxyl radical ($HO^{\cdot}$) through the Fenton reaction (Farrugia and Balzan, 2012). All of these reactive oxygen species can damage bio-molecules including lipids, nucleic acids and proteins, and there is no enzymatic protection against $HO^{\cdot}$ (Farrugia and Balzan, 2012). Furthermore, copper can be toxic even in anaerobic conditions, since it can damage iron–sulphur clusters that are exposed in the structure of proteins, inactivating them (Macomber and Imlay, 2009; Foster et al., 2014).

Organisms have developed complex regulatory mechanisms to maintain a tightly controlled homeostasis of metal ions through the concerted action of transporters, chaperones, sequestering and storage molecules, and metal-responsive transcriptional regulators (Eide, 1998; Ma et al., 2009; O'Halloran and Culotta, 2000). These components allow the organisms to obtain metals ions from different sources under a variety of conditions, distribute the ions to appropriate targets and maintain very low levels of unbound metals in cellular compartments (Eide, 1998; Rae et al., 1999). Disruption of this balance is related to many human diseases, such as Menkes syndrome and Wilson disease, in which the distribution of copper in cells and tissues is impaired (Bleackley and MacGillivray, 2011).

In the yeast *Saccharomyces cerevisiae*, in metal-deficient growth conditions, metal uptake is performed by high-affinity systems, mediated by the copper-responsive transcription factor Mac1 (Keller et al., 2005). On the other hand, in metal-replete conditions,

Abbreviations: Ace1, transcriptional activator in copper-replete cells; Ccc2, copper transporting P-type ATPase; CcO, cytochrome c oxidase; Ccs1, copper chaperone for SOD; Cox1/Cox2, cytochrome c oxidase subunits; Ctr, copper transporter; Fet, ferrous transport; Fre, ferric and cupric reductase; Ftr1, iron permease; ICS, increased copper sensitivity; Mac1, transcriptional activator in copper-deficient cells; MTs, metallothioneins; Rim101, alkaline responsive gene repressor; SOD, copper–zinc superoxide dismutase; YPD, yeast extract peptone dextrose.

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http://dx.doi.org/10.1016/j.fgb.2015.06.007
1087-1845/

metal uptake is regulated by low affinity systems, under control of the transcriptional activator Ace1 (Keller et al., 2005). In the presence of low copper levels, the cell surface iron/copper reductases Fre1 and Fre2 reduce extracellular Cu^{2+} to Cu^{+}, which is then transported across the plasma membrane by the high-affinity copper transporter proteins Ctr1 and Ctr3 (Hassett and Kosman, 1995; Georgatsou et al., 1997; Dancis et al., 1994). High-affinity copper uptake is specific for Cu^{+} over other metals, and it is an energy dependent process which is coupled to K^{+} antiport (Dancis et al., 1994; Lin and Kosman, 1990; De Rome and Gadd, 1987). In presence of high copper levels, copper is transported by the low-affinity copper/iron Fet4 transporter and Smf1, which can also transport Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+} (Hassett et al., 2000; Cohen et al., 2000).

Inside the cell, copper binds to intracellular chaperones responsible for delivering the metal to proteins that use copper as a cofactor. In *S. cerevisiae*, copper is a cofactor in at least three proteins: Fet3 oxidase, cytochrome c oxidase (CcO), and Cu/Zn superoxide dismutase (SOD) Freitas et al., 2003; O'Halloran and Culotta, 2000. The Ccc2 P1-type ATPase is responsible for the transport of copper into Golgi vesicles, where the Fet3 oxidase is copper-metallated (Robinson and Winge, 2010). Chaperones of the Cox family (Cox11 and Cox17) are involved in the delivery of copper to CcO, whereas Ccs1 is the metallochaperone for SOD (Robinson and Winge, 2010). In high copper conditions, the expression of the genes encoding copper-binding metallothioneins (MTs) is upregulated to control the excess of intracellular metal (Thiele, 1988). MTs are low molecular weight cysteine-rich polypeptides, commonly found in eukaryotes, which bind metal ions and are important for their primary storage, transport and detoxification (Hamer et al., 1985; Adamo et al., 2012).

In yeast, most of the excess metal ions are stored in the vacuole, a dynamic organelle that is critical for the maintenance of intracellular pH, and functions as a storage compartment for amino acids, metal ions, and other molecules (Klionsky et al., 1990). It has been proposed that the vacuole is as important as MTs for copper detoxification (Eide et al., 1993). In copper deprivation conditions Fre6 and Ctr2, present in the vacuolar membrane, mediate the reduction and transfer, respectively, of copper from the lumen of the vacuole to the cytosol (Cyert and Philpott, 2013).

Copper and iron homeostasis are both influenced by environmental pH, since the maintenance of a proton gradient through the plasma membrane H-ATPase, which extrudes protons, is important for the uptake of nutrients and cations (Serrano et al., 2004). Many genes involved in iron and copper uptake and transport (*FET3*, *FRE1*, *FRE3*, *FRE4*, *FET4*, *CTR1*, *CTR3*) are upregulated in alkaline pH stress (Arino, 2010). Iron and copper are limiting factors for growth in pH stress. In fact, the addition of these metals in micromolar concentrations improves growth at alkaline pH (Serrano et al., 2004). In *S. cerevisiae*, Rim101 is a transcriptional repressor in alkaline pH stress, inhibiting the expression of other transcriptional repressor genes, such as *NRG1*, which is related to ion homeostasis and alkaline pH protection, and *SMP1*, related to sporulation and invasive growth (Lamb and Mitchell, 2003).

The gene *YJL077C* was denoted as *ICS3* (for "increased copper sensitivity") in a high throughput study performed with 150 deletion mutants for genes of unknown function (Entian et al., 1999). *ICS3* has also been indirectly implicated in the process of vacuolar homeostasis. It was identified in a screen study as a gene related to sensitivity to Sortin 2, a synthetic compound that affects protein targeting to the vacuole in *S. cerevisiae* (Norambuena et al., 2008). Additionally, the *Δics3* strain is sensitive to hydrochloric and acetic acids (Kawahata et al., 2006), and possesses abnormal vacuole morphology (Michaillat and Mayer, 2013). Our group identified *ICS3* in a screen for genes of unknown function which were sensitive to oxidative stress (Costa and Monteiro, 2014).

The objective of the present study is to explain the oxidant and copper sensitivities of the mutant strain for the *ICS3* gene. Here, we show that the *Δics3* strain is sensitive to oxidative stress because these cells have copper concentration three times higher than the wild-type strain in optimal growth conditions. High copper concentration exposure in acidic conditions also leads these cells to accumulate intracellular copper and iron. Furthermore, *Northern blot* analyses indicate that *ICS3* is regulated by Rim101 from the pH-dependent regulon, rather than by the copper-dependent transcription factors Mac1 and Ace1. Together, the data indicate that the *ICS3* gene is involved in copper homeostasis in a pH-dependent manner, impacting the antioxidant response.

2. Material and methods

2.1. Yeast strains

S. cerevisiae wild-type strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *Δics3* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ICS3::KANMX4*), *Δics1* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ICS1::KANMX4*), *Δace1* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ACE1::KANMX4*), *Δrim101* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM101::KANMX4*), and *Δmac1* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MAC1::KANMX4*) were obtained from Yeast Knockout (YKO) Deletion Collection (Invitrogen, Carlsbad – CA, USA).

2.2. Sensitivity to hydrogen peroxide

Cells were pre-inoculated in YPD culture media (yeast extract 1%, peptone 2% and glucose 2%) and grown at 30 °C overnight. The cultures were diluted to an initial OD_{600} of 0.2 and grown in fresh YPD until logarithmic phase (OD_{600} 0.6–1.0). An aliquot of cells was diluted in water to OD_{600} of 0.2, and four subsequent 1:5 dilutions were performed; 5 μL drops of each dilution were spotted onto YPD plates containing no (control) or 3 mM hydrogen peroxide (Merck, Millipore, Billerica – MA, USA). Growth was recorded after 24 h incubation at 30 °C. The figure shown (Fig. 1) is representative of at least three independent experiments.

2.3. Sensitivity to metal ions

Cells pre-inoculated in YPD were grown at 30 °C overnight. The cultures were diluted to OD_{600} of 0.2 in fresh liquid YPD containing no (control), 7 mM or 10 mM copper sulphate and grown at 30 °C for the time indicated in the figure legends. Cells were then diluted in water to OD_{600} of 0.2, and four subsequent 1:5 dilutions were performed; 5 μL drops of each dilution were spotted onto YPD plates. Tests with cobalt chloride and iron sulphate were performed similarly. When necessary, the pH of the culture media was adjusted to 4.0 with the addition of HCl, or to 6.0 with the addition of NaOH immediately before the first dilution to OD_{600} of 0.2 in fresh liquid media. In all sensitivity tests, growth was recorded after 24 h of incubation at 30 °C. The figures are representative of at least three independent experiments.

2.4. Growth curves

Cells were grown at 30 °C in YPD overnight, and then diluted to OD_{600} of 0.1. After dilution, the pH of the media was adjusted to 4.0 or 6.0 when necessary and 10 mM copper sulphate was added or not (control). After these additions the final pH of the culture was measured. The growth was followed spectrophotometrically (UNICO SQ4802 Double Beam Spectrophotometer – NJ – USA) for 75 h by measuring the OD_{600} at different time intervals. The curve

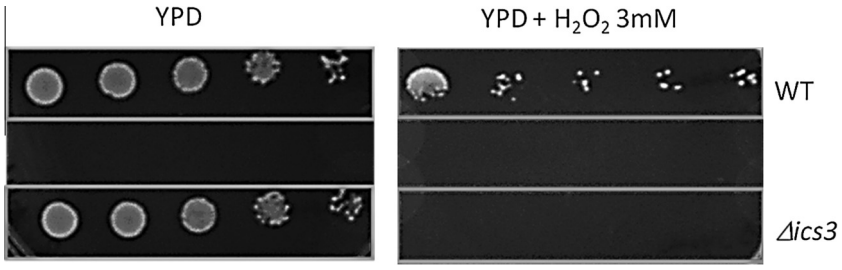

Fig. 1. Sensitivity to hydrogen peroxide. The wild-type strain BY4741 and the mutant *Δics3* were grown in YPD medium pH 6.0 at 30 °C for 24 h. The cultures were then diluted to an OD_{600} of 0.2, and 5 times serial dilutions were grown in YPD plates pH 6.0 containing no or 3 mM of hydrogen peroxide. Growth was monitored after 24 h at 30 °C. This image represents at least three independent experiments.

represents the mean (with standard deviation bars) of three biological replicates. Curve fitting and statistical analyses were performed with software GraphPad Prism version 6.05.

2.5. Metal content

The experiments were performed following the protocol described in Schuller et al. (2013) with modifications (Schuller et al., 2013). Briefly, cells were grown at 30 °C in YPD for 20 h, and then diluted to OD_{600} of 0.6. Copper sulphate was added or not to the YPD medium at 10 mM concentration and the pH was adjusted to 4.0 with HCl, or to 6.0 with NaOH. After growth in the absence or presence of copper for 20 h at 30 °C, the cells were harvested by centrifugation, the cell pellet was washed with deionized sterile water and the supernatant was discarded (repeated twice). The remaining pellets were dried under vacuum for 20 h at 30 °C in a Speed Vac Concentrator plus (Eppendorf, Hamburg – Germany). After weighing, the samples were digested with concentrated nitric acid in a block digester until complete solid dissolution. The iron and copper content of each sample was analysed at the Analytical Centre of the Institute of Chemistry at the University of São Paulo in an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) Spectro Ciros (Kleve, Germany), model Arcos, CCD equipment. Iron was analysed at 259.941 nm, and copper at 324.754 nm. The amounts given in the figures are the means and standard deviations of three independent experiments. Statistical analyses were performed using software GraphPad Prism version 6.05, through unpaired *t*-student test, with $p < 0.05$.

2.6. ICS3 regulation

The wild-type strain BY4741 and the mutants *Δics3*, *Δrim101*, *Δace1* and *Δmac1* were grown in YPD media overnight at 30 °C. The cultures were diluted to OD_{600} 0.2 in fresh YPD media at pH 4.0 and 6.0 in the presence or absence of 0.5 mM of copper sulphate, and grown until mid-logarithmic phase (OD_{600} between 0.8 and 1.4). Cells were washed with 50 mM EDTA in deionized sterile water, and harvested by centrifugation. Total RNA of each sample was obtained by the hot acid phenol method (Ausubel et al., 1994), and separated through electrophoresis formaldehyde–agarose gels. The separated RNAs were transferred to a positively charged membrane (Amersham Hybond™ – N^+) by capillary blotting and fixed by UV exposure for 2 min. Pre-hybridization, *ICS3* and *CCC2* DNA labelling, hybridization and membrane washing were performed with the Amersham Gene Images AlkPhos Direct Labelling and Detection System kit, according to manufacturer's manual instructions without modifications. Detection was carried out using the NBT/BCIP Reagent Kit from Invitrogen for 24 h of incubation.

3. Results

In order to identify the still uncharacterised viable mutants (total of 217 selected strains), whose genes could confer resistance to oxidative stress in *S. cerevisiae*, the cell viability of the knockout strains from the YKO Deletion Collection was tested in the presence of hydrogen peroxide (Costa and Monteiro, 2014). Among the mutants tested, six strains were found to have higher sensitivity to hydrogen peroxide; one was the knockout for the gene *YJL077C/ICS3* (*Δics3*). This experiment was reproduced focusing on the *Δics3* mutant, and the sensitivity of these cells to oxidative stress induced by hydrogen peroxide at 3 mM concentration was confirmed (Fig. 1).

Previously, the mutant *Δics3* was described as sensitive to copper in a screen of 150 deletion mutants for genes of unknown function (Entian et al., 1999). To characterise the sensitivity of the *Δics3* strain to metals, we performed viability tests in the presence of copper, cobalt and iron (Fig. 2). *Δics3* cells were not sensitive to cobalt chloride or iron sulphate at either of the concentrations tested (7 mM and 10 mM). At 7 mM copper sulphate, both wild-type and *Δics3* strains behaved similarly. However, at 10 mM concentration, *Δics3* cells were more sensitive to copper exposure than the wild-type strain (Fig. 2). Furthermore, incubation in the presence of copper for only 4 h resulted in lower viability of *Δics3* cells, whereas 8 h of incubation with cobalt and iron did not induce sensitivity. This indicates that, of all the metals tested, *ICS3* is likely to be specifically related to copper homeostasis.

It has also been suggested that *Δics3* cells are sensitive to hydrochloric and acetic acids (Kawahata et al., 2006). Considering that the addition of copper sulphate could change the pH of the culture medium, the pH of all media was measured before cell inoculation. YPD medium initially presented pH 6.0, however, the addition of 10 mM copper sulphate decreased the pH to around 4.0. This raises the possibility that the sensitivity of *Δics3* cells in the presence of copper sulphate was due to the acidic pH, rather than to the metal.

To clarify this issue, we constructed growth curves for the wild-type and *Δics3* strains in the absence and presence of copper (10 mM) at pH 4.0 (Fig. 3A) and 6.0 (Fig. 3B). In these experiments, pH 4.0 was reached either with the addition of 10 mM copper sulphate or with the addition of HCl in the absence of copper sulphate. In cultures with copper sulphate, pH 6.0 was reached with the addition of NaOH. At pH 4.0, the growth curve of wild-type and *Δics3* cells was similar in the absence of copper (Fig. 3A). In the presence of 10 mM of copper sulphate at pH 4.0, both wild-type and *Δics3* strains grew at a slower rate when compared to the curve in the absence of copper. Interestingly, after 48 h, the growth curve of the wild-type and *Δics3* strains diverged significantly: the wild-type strain recovered and began to grow faster, while the *Δics3* mutant continued to grow slowly. Indeed, we also observed

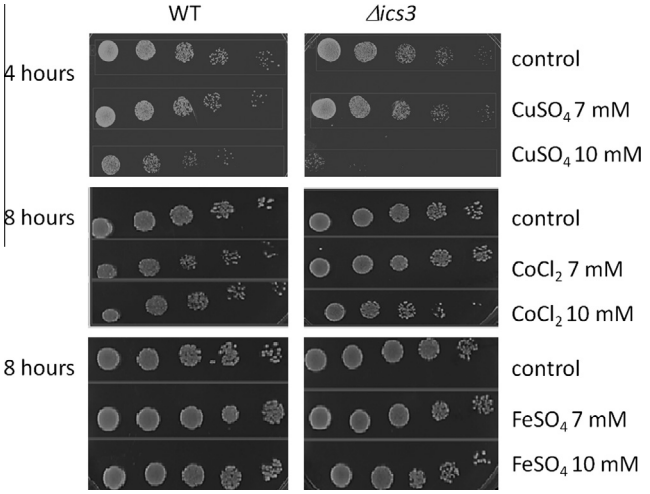

Fig. 2. Sensitivity to metals. The wild-type strain BY4741 and the mutant *Δics3* were grown in YPD medium in the absence (control) or, presence of metals at 7 mM or 10 mM concentration, as indicated. After 4 h ($CuSO_4$) or 8 h ($CoCl_2$ and $FeSO_4$) of incubation at 30 °C (the pH of the media was not determined), the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. Growth was monitored after 24 h at 30 °C and the image is representative of three replicates.

that the wild-type strain recovered its cell viability, forming colonies in solid fresh media, while the *Δics3* strain did not (data not shown). Unexpectedly, when the pH was adjusted to 6.0, the presence or absence of copper did not affect the growth of either the wild-type or the *Δics3* strains. Both grew at similar rates as in the absence of copper at pH 4.0 (Fig. 3B).

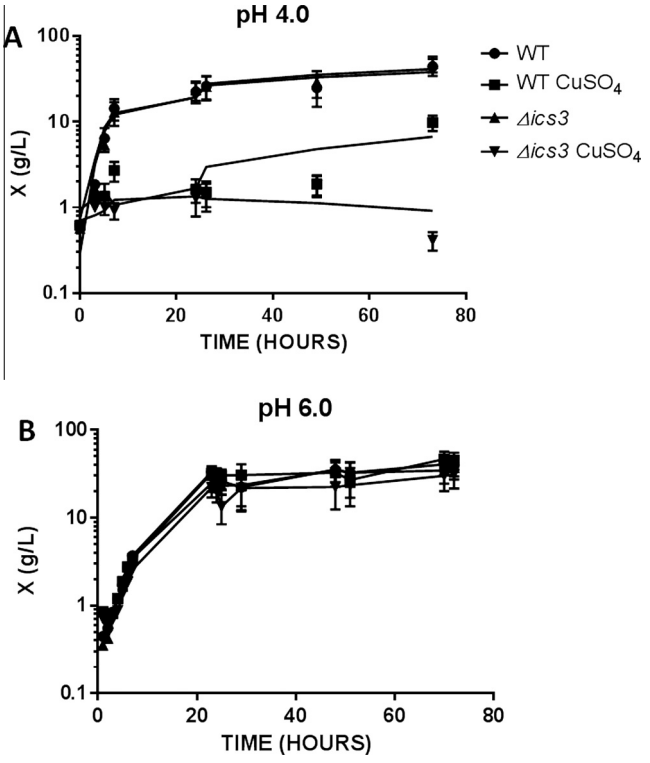

Fig. 3. Growth curves for the wild-type strain BY4741 and the mutant *Δics3* in the absence or presence of copper (10 mM) at pH 4.0 (A) and pH 6.0 (B). The cells were grown in YPD pH 6.0 at 30 °C. Mean and standard deviation of three experiments performed independently.

To better characterise this pH dependence, we also carried out viability tests in which we challenged cells with separate stressors, at pH 4.0 in the absence of copper (Fig. 4A), and at pH 6.0 in the presence of 10 mM of copper sulphate (Fig. 4B). In the absence of copper, the acidic pH did not impair the growth of either wild-type or *Δics3* strains (Fig. 4A). Additionally, in the presence of 10 mM of copper sulphate, when the pH was adjusted to 6.0, both wild-type and *Δics3* strains grew at the same rate as in the control without copper sulphate (Fig. 4B). These results indicate that the cellular response to copper depends on low pH, since the sensitivity of the *Δics3* mutant was not observed in pH 6.0. Despite the suggestion that *Δics3* cells are sensitive to hydrochloric and acetic acids, the present data indicate they are not sensitive to pH 4.0 in the absence of copper. One possible explanation for this apparent inconsistency is that previous experiments reached lower pH values (2.6 with hydrochloric acid and 3.3 with acetic acid) (Kawahata et al., 2006).

To investigate the dependence of copper sensitivity on pH, the knockout for the gene *YIR004W/ICS1* (*Δics1*) was also analysed, since this strain had been shown to be even more sensitive to copper than the *Δics3* mutant (Entian et al., 1999). We performed viability tests for *Δics1* cells after 8 h of incubation in the presence of 7 mM or 10 mM of copper sulphate at pH 4.0 (Fig. 5). The *Δics1* strain was significantly more sensitive to copper at pH 4.0 than *Δics3*, since it suffered even with 7 mM of copper sulphate, whereas *Δics3* suffered only at 10 mM concentration. When the pH was adjusted to 6.0, however, the *Δics1* strain behaved identically to the wild-type (Fig. 5). These results indicate that both *Δics1* and *Δics3* strains are copper-sensitive only in acidic conditions.

One possible explanation for the sensitivity of the *Δics3* strain to copper and oxidant exposure could be an increase in the accumulation of intracellular copper. Higher levels of copper could increase the ratio of free metals that can catalyse the generation of toxic levels of reactive oxygen species through Fenton's reaction, or impair the activity of proteins dependent on iron–sulphur clusters. Additionally, copper accumulation may depend on pH, since acidic conditions have been shown to induce genes involved in metal metabolism and homeostasis in yeast (Kawahata et al., 2006).

To analyse the concentration of copper in wild-type and *Δics3* cells, we performed atomic emission spectroscopy experiments with cells previously incubated with 10 mM of copper sulphate and with control cells. The copper content in wild-type cells in control conditions (grown only in YPD) was significantly different at pH 4.0 and pH 6.0 (Fig. 6A), with three times more copper in acidic pH. The difference in copper content for *Δics3* cells at pH 4.0 and 6.0 was not significant (Table 1). However, *Δics3* cells at both pH values accumulate the same amount of copper as wild-type cells at pH 4.0 (Fig. 6A), indicating that the *Δics3* strain has abnormal copper homeostasis even in neutral conditions. This threefold greater amount of copper at pH 6.0 for *Δics3* cells in comparison to wild-type cells is consistent with their sensitivity to oxidant exposure in optimal growth conditions (Fig. 1).

When the cells were incubated with 10 mM of copper sulphate before metal content determination (Fig. 6B), the levels of copper for both wild-type and *Δics3* strains were similar at pH 6.0, whereas at pH 4.0, the levels of copper for the *Δics3* strain were higher than those of the wild-type (Fig. 6B, Table 1). These results are in agreement with those from the viability tests, since the bioaccumulation of copper was greater at lower pH, especially for *Δics3* cells, leading to copper sensitivity.

Copper and iron homeostasis are linked: in the cell membrane, both iron and copper are reduced by iron/copper reductases before uptake, and the high-affinity iron uptake system requires the copper containing oxidase Fet3 (Freitas et al., 2003; Rae et al., 1999). In order to verify whether *ICS3* deletion influences iron homeostasis,

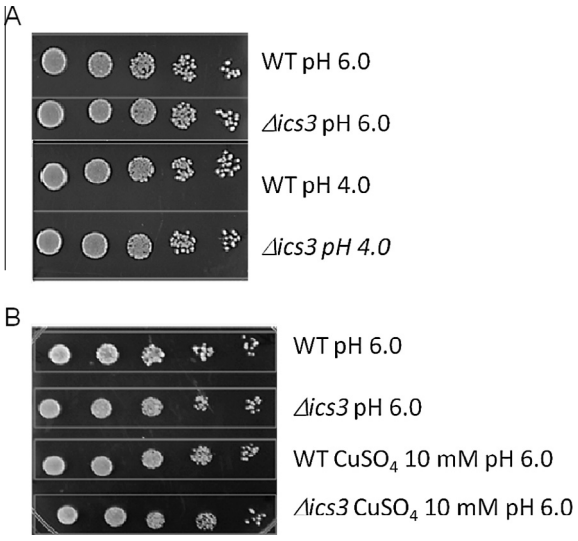

Fig. 4. Sensitivity to acidic pH. (A) Wild-type and *Δics3* strains were grown in YPD medium with pH adjusted to 4.0 with HCl. After 8 h, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. (B) Wild-type and *Δics3* strains were grown in YPD medium in the absence (control) or presence of 10 mM of $CuSO_4$. The pH of the medium was adjusted to 6.0 with NaOH. After 8 h of incubation at 30 °C, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. The image is representative of at least three independent experiments.

we also determined the iron content. Iron levels were always higher at pH 4.0 than at pH 6.0 (Fig. 6C and D), however, the ability to accumulate iron in acidic pH was impaired in *Δics3* in control conditions (Fig. 6C – Table 2). Interestingly, the iron accumulation of the *Δics3* strain with respect to the wild-type varied in different pH conditions. At pH 6.0, the *Δics3* strain had less than half of the content of the wild-type after copper treatment (Fig. 6C and D). At pH 4.0, on the other hand, the iron concentration of *Δics3* treated cells was almost 30% greater than the wild-type (Fig. 6D). These results indicate that the role of *ICS3* in copper homeostasis is dependent on the pH; this may directly or indirectly influence iron homeostasis. All these features together make mutant cells sensitive to oxidant agents.

Because *ICS3* appears to be involved in copper homeostasis in a pH-dependent manner, we carried out a *Northern blot* analysis to determine whether the expression of *ICS3* is regulated by copper-dependent transcription factors, such as Ace1 or Mac1, or by Rim101, which is involved in the response to alkaline pH stress (Mira et al., 2009). *ICS3* expression is induced at acidic pH in wild-type, and this increase is more pronounced in *Δrim101*. In relation to copper exposure, we did not observe a clear effect of the copper-related transcriptional factors Ace1 and Mac1 (Fig. 7).

Because the pH and copper affect the vacuole, and previous results have shown *ICS3* has a role in protein trafficking Golgi–endo some–vacuole (Norambuena et al., 2008; Copic et al., 2009), we also studied the expression of the gene encoding the copper transporting P1-type ATPase, *CCC2*. *CCC2* gene expression was increased in *Δics3* and *Δrim101* cells only at pH 4.0. Interestingly, *CCC2* expression in *Δics3* at pH 4.0 is slightly higher in the presence of copper than in its absence. This suggests a close relationship between *ICS3* and *CCC2* expression.

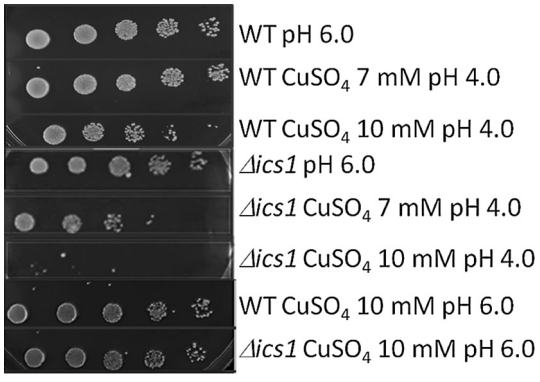

Fig. 5. Sensitivity to copper is dependent on pH. The wild-type and *Δics1* strains were grown in YPD media in the absence (control), or presence of 7 mM or 10 mM of $CuSO_4$, as indicated. In the last two lines at the bottom of the figure, the pH of the medium was adjusted to 6.0 with the addition of NaOH. After 8 h of incubation at 30 °C, the cultures were diluted to OD_{600} of 0.2 and 5 times serial dilutions were grown in YPD plates, pH 6.0. The images are representative of at least three independent experiments.

4. Discussion

Although the genome of *S. cerevisiae* was sequenced 19 years ago, the biological and molecular functions of more than 20% of its ORFs ("open reading frames") remain unknown. The *ICS3* gene was identified in an initiative to characterise the still unknown oxidative stress responsive genes in yeast (Costa and Monteiro, 2014). Prior to this study, the function of *ICS3* was poorly characterised in wide scale genes studies (Entian et al., 1999; Norambuena et al., 2008; Kawahata et al., 2006; Copic et al., 2009). This paper presents a more detailed characterisation of the function of *ICS3* and an investigation of the increased sensitivity of the mutant strain to copper exposure.

We first observed that *Δics3* cells show increased sensitivity to hydrogen peroxide (Fig. 1). Our data also indicate that *ICS3* is involved in copper homeostasis, and more specifically, that *Δics3* cells are sensitive to copper only in acidic conditions. We also observed pH-dependent copper sensitivity in wild-type and *Δics1* strains, suggesting that this is a common yeast cell phenomenon. However, it is important to consider that in pH 4.0, copper sulphate solubility is higher than in pH 6.0, turning the copper availability increased to yeast in acidic culture media (Gadd and Griffiths, 1978). This can explain the data observed in wild-type strain (and perhaps *Δics1*, but it was not analysed), however *Δics3* cells accumulate more copper even in the lowest availability condition (without pre-treatment with copper and in pH 6.0, Fig. 6A).

In this regard, it was observed that the accumulation of both copper and iron is greater in acidic conditions. This result agrees with a recent study aimed at identifying genes related to copper homeostasis, which showed enrichment for the Gene Ontology (GO) processes "Golgi to vacuole transport and vacuolar acidification" (Schlecht et al., 2014). These authors also observed that when respiratory growth is impaired by alkaline media, the growth ratio can be rescued by the addition of copper sulphate (Schlecht et al., 2014). Together, the data suggest that the relationship of the copper response to pH may be mediated by vacuolar processes.

We note that deletion of the *ICS3* gene leads to an impairment in copper homeostasis and in the ability of yeast to increase iron to wild-type levels in acidic conditions (Fig. 6 and Table 2). At pH 6.0, *Δics3* cells possess a threefold higher endogenous copper content than wild-type cells. This explains why these cells are more sensitive to oxidative exposure, since there is more copper available to participate in Fenton's reaction (Farrugia and Balzan, 2012) and to damage iron–sulphur clusters in proteins (Macomber and Imlay, 2009; Foster et al., 2014).

Under exposure to high copper concentrations at pH 4.0, *Δics3* cells accumulated higher copper levels than the wild-type. At pH 6.0, conversely, the levels of copper were the same for both

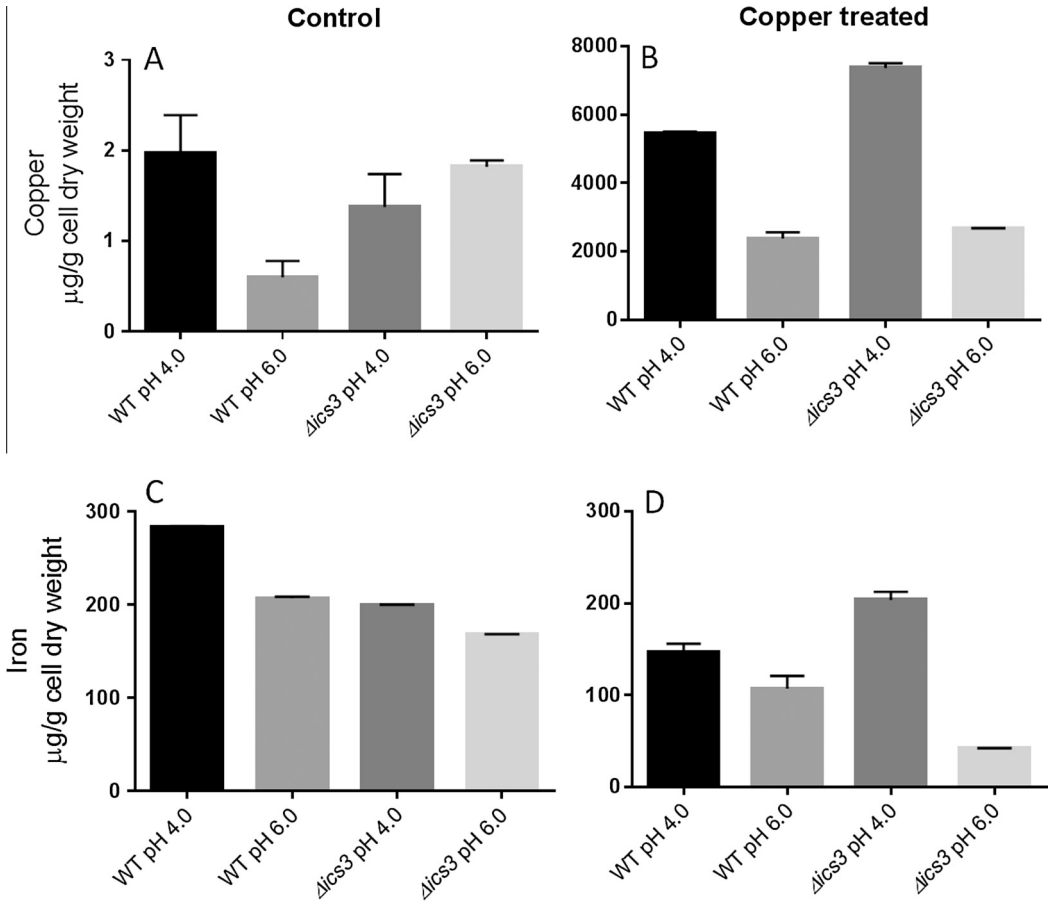

Fig. 6. Atomic emission spectroscopy analysis of copper (A and B) and iron (C and D) contents of wild-type and *Δics3* strains at pH 4.0 or 6.0, previously incubated in the absence (A and C) or presence of copper sulphate (B and D) for 20 h. Mean and standard deviation of three experiments performed independently.

Table 1
Statistical analyses of the copper content difference between the strains tested.

	Unpaired test *t* significant	$p < 0.05$
Control conditions	pH 4.0	pH 6.0
WT × WT	Y, $p = 0.00062$	
Δics3 × *Δics3*	N	
WT × *Δics3*	N	Y, $p = 0.0004$
Copper treated		
WT × WT	Y, $p < 0.0001$	
Δics3 × *Δics3*	Y, $p < 0.0001$	
WT × *Δics3*	Y, $p < 0.0001$	N

Y = yes, N = no.

wild-type and *Δics3* strains after incubation with copper. We observed that the viability and the growth ratio of *Δics3* cells decreased only in acidic conditions, which is consistent with the metal accumulation we reported. It is important to note that the vacuole uptake of metal ions is carried out by H^+/ion antiport systems (Klionsky et al., 1990), suggesting that when H^+ concentration is high, the vacuole contains abundant protons that impair the antiport of copper, resulting in less vacuolar copper storage. The vacuole morphology of *Δics3* cells has already been characterised as abnormal (Michaillat and Mayer, 2013).

Copper is known to be required for iron homeostasis in yeast, plants and mammals (Freitas et al., 2003). *S. cerevisiae* takes up iron from the environment through multiple mechanisms. One of these,

Table 2
Statistical analyses of the iron content difference between the strains tested.

	Unpaired test *t* significant, $p < 0.05$	
Control conditions	pH 4.0	pH 6.0
WT × WT	Y, $p < 0.0001$	
Δics3 × *Δics3*	Y, $p < 0.0001$	
WT × *Δics3*	Y, $p < 0.0001$	Y, $p < 0.0001$
Copper treated		
WT × WT	Y, $p = 0.0139$	
Δics3 × *Δics3*	Y, $p < 0.0001$	
WT × *Δics3*	Y, $p = 0.0014$	Y, $p = 0.0012$

Y = yes, N = no.

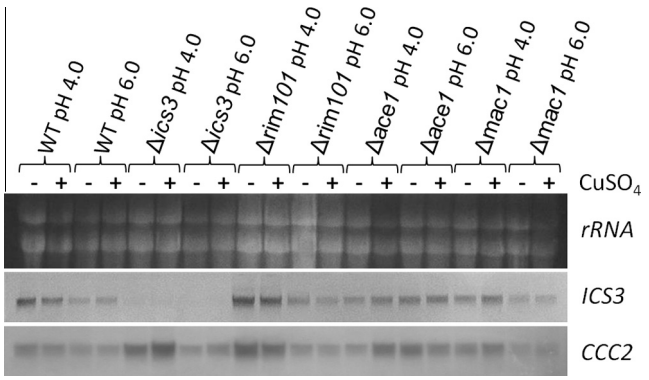

Fig. 7. *ICS3* regulation. Northern blot analysis of *ICS3* and *CCC2* in cells grown in YPD at pH 4.0 or pH 6.0, in the presence (+) or absence (−) of 0.5 mM of $CuSO_4$. The image shows ribosomal RNA (rRNA) as loading reference.

the high-affinity iron uptake system, is active in iron deprivation conditions and requires the copper-containing oxidase Fet3, which oxidises Fe^{2+} to Fe^{3+} which, in turn, is transported through the plasma membrane by the Ftr1 permease (Askwith et al., 1994; Stearman et al., 1996). Our results show that at pH 6.0, non-treated *Δics3* cells accumulate more copper but less iron than wild-type cells. At pH 6.0, both wild-type and *Δics3* cells incubated with copper accumulate more copper, but, again, less iron than in non-treated cells, especially for the *Δics3* strain (Fig. 6). One possible explanation for the lower iron levels under copper-replete conditions is the competition between copper and iron ions for the low-affinity iron transport protein Fet4, which may also transport metals other than iron (Dix et al., 1994). Additionally, iron and copper are both substrates for the reductases Fre1 and Fre2 in *S. cerevisiae*, which reduce Fe^{3+} and Cu^{2+} before they are transported through the plasma membrane by their respective transporters (Freitas et al., 2003). Nevertheless, metals in excess impair metalloenzyme functions by competing with and replacing the optimal metal cofactors (Argüello et al., 2012). There should be a balance: copper is necessary for iron uptake, but if the copper concentration is too high, there should be competition between copper and iron for Fre1/Fre2 and Fet4 proteins, decreasing iron uptake.

In acidic conditions, the profile is different from that described for neutral conditions. Non-treated wild-type cells accumulate more copper and iron at pH 4.0 than *Δics3* cells. However, when incubated with copper at pH 4.0, *Δics3* treated cells accumulate more copper and iron than wild-type cells. The higher metal levels observed in acidic conditions are possibly related to a higher electrochemical potential in the vacuolar membrane at lower pH (Cyert and Philpott, 2013). It was shown that yeast cells with mutations in genes essential for vacuolar structure or acidification have defects in copper detoxification, and that normal vacuolar function and integrity are necessary to retain normal iron and copper homeostasis in yeast (Szczypka et al., 1997).

Our data show that *ICS3* copper response depends directly on pH. Therefore, we also examined the transcriptional regulation of *ICS3* in response to alterations in copper concentration and pH (Fig. 7). We observed that pH is the main determinant of the transcriptional response, since the expression of *ICS3* was induced in acidic pH in wild-type cells and enhanced with deletion of *RIM101*, the alkaline-responsive transcriptional repressor. We did not observe changes in transcription with 0.5 mM of copper in rich medium. Lower doses of copper were used to preserve cell viability for mRNA analyses. We also tested the transcriptional activators responsive to copper Ace1 and Mac1, but we did not observe a clear effect of these factors on *ICS3* expression. In summary, the extracellular pH is a critical factor regulating the expression of this gene.

The description of the *ICS3* gene in *Saccharomyces Genome Database* (SGD: http://www.yeastgenome.org/locus/S000003613/overview), based on Copic et al. (2009) and Norambuena et al. (2008), suggests that the protein encoded by this gene is involved in secretory protein trafficking (Golgi–endosome–vacuole). A potential candidate to connect cooper and pH to protein vacuolar sorting is the Ccc2 P1-type ATPase which uses copper, increased at acidic pH (Fig. 6A), to metallate the multicopper oxidase Fet3 into Golgi apparatus, rendering its secretion to extracellular media. For this reason, we also analysed the expression pattern of the *CCC2* gene (Fig. 7). Interestingly, *CCC2* gene expression increases in *Δics3* cells, suggesting a close relationship between their metabolic functions. *CCC2* and *ICS3* expression patterns are similar in response to *RIM101*, since both are induced in *Δrim101* cells only at acidic pH. Again, the presence of copper was not a critical regulator of *CCC2* expression, although it seems slightly increased in *Δics3* and *Δace1* cells exposed to copper at pH 4.0.

The present data demonstrate that *Δics3* cells are sensitive to cooper only in acidic extracellular pH, due to, at least, two important effectors: Ccc2 and Rim101. Ccc2 is augmented in *Δics3* cells, which can lead to enhanced post-translational modification of the metal oxidase Fet3 that is secreted. This increases the amount of substrate available to the transmembrane permease Ftr1, increasing the activity of the high affinity metal transport system, leading to the observed metal accumulation. Furthermore, *CCC2* and *ICS3* present similar expression profiles in response to pH and to Rim101 regulon. Ccc2p is described as a copper exporter from the cytosol and is necessary for uptake of iron, which is important for metal homeostasis (Fu et al., 1995).

In conclusion, the data presented suggest an intersection between Ccc2 and Ics3 pathways. Additional experiments, such as the investigation of the sensitivity to copper in the double mutant *Δics3Δccc2*, or complementation of function between these genes, could be helpful to elucidate this hypothesis. Clearly, a complex cellular mechanism involving vacuolar conditions, pH, and *ICS3* is critical for maintaining the interconnected homeostasis of iron and copper in yeast.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank to Prof Dr Luiz Carlos Martins das Neves for technical support and advice. Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Grant 2009/01303-1) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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